FEBS Letters 580 (2006) 6275–6280

Mass spectrometry studies of demetallation of haemin by recombinant horse L chain apoferritin and its mutant (E 53,56,57,60 Q)

Natalia de Val[a,*], Haiko Herschbach[b], Noëlle Potier[b], Alain Van Dorsselaer[b], Robert R. Crichton[a]

[a] *Department of Biochemistry, Université Catholique de Louvain, Bâtiment Lavoisier, 1 Place Louis Pasteur, 1348 Louvain-la-Neuve, Belgium*
[b] *Bio-Organic Mass Spectrometry Laboratory, Université Louis Pasteur, Bâtiment R5, 25 rue Becquerel, F67087 Strasbourg Cedex 2, France*

Received 11 September 2006; accepted 11 October 2006

Available online 24 October 2006

Edited by Miguel De la Rosa

Abstract **An essential difference between eukaryotic ferritins and bacterioferritins is that the latter contain naturally, in vivo haem as Fe-protoporphyrin IX. This haem is located in a hydrophobic pocket along the 2-fold symmetry axes and is liganded by two Met 52.**

However, in in vivo studies, a cofactor has been isolated in horse spleen apoferritin similar to protoporphyrin IX; in in vitro experiments, it has been shown that horse spleen apoferritin is able to interact with haem. Studies of haemin (Fe(III)-PPIX) incorporation into horse spleen apoferritin have been carried out, which show that the metal free porphyrin is found in a corresponding pocket to haem in bacterioferritins [Précigoux, G., Yariv, J., Gallois, B., Dautant, A., Courseille, C. and Langlois, d'Estaintot B. (1994) A crystallographic study of haem binding to ferritin. Acta Cryst. D 50, 739–743].

A mechanism of demetallation of haemin by L-chain apoferritin was proposed [Crichton, R.R., Soruco, J.A., Roland, F., Michaux, M.A., Gallois, B., Précigoux, G., Mahy, J.P. and Mansuy. (1997) Remarkable ability of horse spleen apoferritin to demetallate hemin and to metallate protoporphyrin IX as a function of pH. J. P. Biochem. 36, 49, 15049–15054]: this involved four Glu residues (53,56,57,60) situated at the entrance of the hydrophobic pocket and appeared to be favoured by acidic conditions.

To verify this mechanism, we have mutated these four Glu to Gln and examined demetallation in both acidic and basic conditions.

In this paper, we report the mass spectrometry studies of L-chain apoferritin and its mutant incubated with haemin and analysed after different times of incubation: 15 days, 2 months, 6 months, 9 months and 12 months. These studies show that the recombinant L-chain apoferritin and its mutant are able to demetallate haemin to give a hydroxyethyl protoporphyrin IX derivative in a dimeric form [Macieira, S., Martins, B. M. and Huber, R. (2003) Oxygen-dependent coproporphyrinogen IX oxidase from *Escherichia coli*: one-step purification and biochemical characterization. FEMS. Microbiology Letters 226, 31–37].

Keywords: Ferritin; Bacterioferritin; Demetallation; Haemin; Protoporphyrin IX; Mass spectrometry

1. Introduction

Iron, element 26 in the periodic table, is the second most abundant metal (after aluminium), and the fourth most abundant element in the Earth's crust [1]. It is an essential bioelement which participates in many biological systems in mammals, plants and bacteria.

Most of the iron present in living organisms is complexed in proteins, although it may also be present in a soluble pool of low molecular weight complexes [2]. Uncomplexed iron together with superoxide (which reduces Fe(III), {1}) and hydrogen peroxide (which is decomposed by the Fenton reaction, {2}) [3] provides a lethal mixture generating reactive hydroxyl radicals. The sum of these reactions is the Haber–Weiss reaction {3} [4]

$$Fe^{3+} + O^{2-\cdot} \rightarrow Fe^{2+} + O_2 \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot} \quad (2)$$

$$O^{2-\cdot} + H_2O_2 \rightarrow OH^- + OH^{\cdot} + O_2 \quad (3)$$

The hydroxyl radical is very reactive causing lipid peroxidation, DNA and protein damage and degradation of other biomolecules [5].

Sequestration of iron in a soluble, bioavailable and non-toxic form is achieved by iron storage in proteins, ferritins and bacterioferritins, which are widely distributed throughout in living kingdom. Different kinds of ferritins have been isolated from vertebrates, invertebrates, flowering plants, fungi and bacteria. They can be classified into ferritins containing only non-haem iron, like mammalian, plant and certain bacterial ferritins, and on the other hand, bacterioferritins containing both non-haem and haem-iron (bacterioferritins in *E. coli*, *A. vinalendii*, etc.). There is an extensive structural homology between all these ferritins (molecular weight, amino acid composition, isoelectric point, size, tertiary and quaternary structure) [1,6].

Ferritins are tetracosameric protein shells (24-mers) with their subunits packed in 432 symmetry, each of molecular weight around 20 kDa, forming a hollow protein shell of nearly 80 Å in diameter. The tetracosamers of molecular weights ranging from 450 to 500 kDa, and are capable of storing up to a maximum of 4500 Fe(III) atoms as an inorganic oxyhydroxide complex, which in mammalian ferritins ressembles the biomineral ferrihydrite [7] (see Fig. 1).

Mammalian ferritins contain two subunit types, H and L [8,9], which despite extensive differences in their amino acid sequences have essentially identical tertiary conformations,

*Corresponding author. Fax: +32 10 47 27 96.
E-mail address: deval@bioc.ucl.ac.be (N. de Val).

0014-5793/$32.00
doi:10.1016/j.febslet.2006.10.034

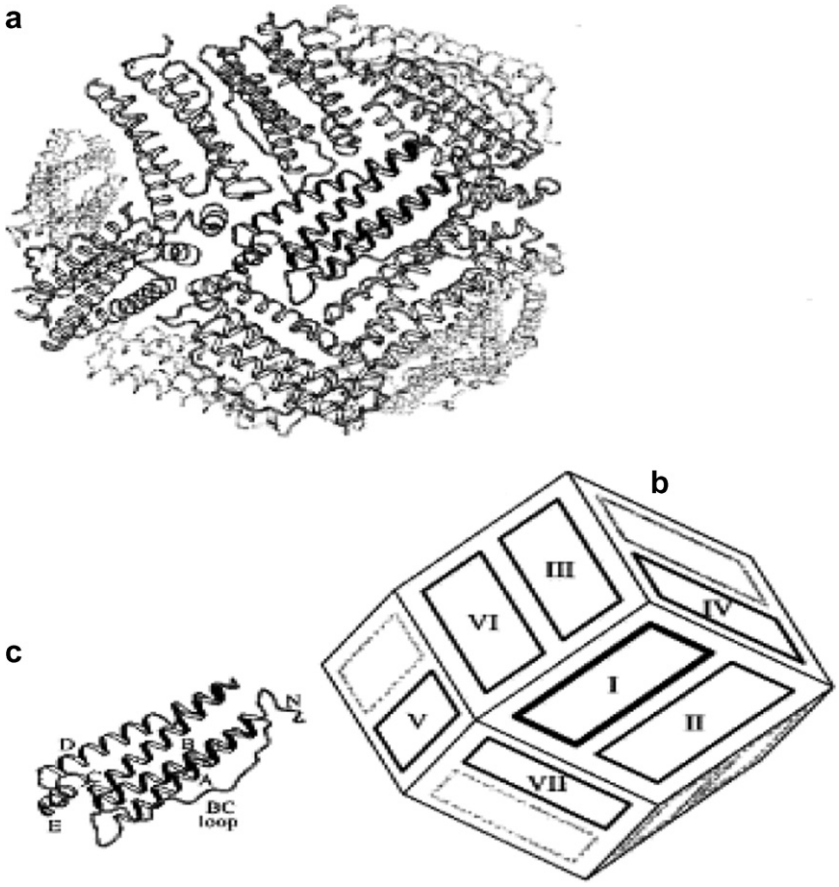

Fig. 1. (a) Overview of a ferritin molecule showing the relative positions and interfaces between symmetry related subunits. (b) Labelling scheme of symmetry related subunits. (c) Detail of a single ferritin subunit [16].

structurally organized into five α helices of which four (A, B, C and D) form a compact four helical bundle. The last short helix E is placed almost perpendicular to the other four α helices. The four long helices (A, B, C and D) form a tight bundle which does not have a uniformly hydrophobic interior. B and D helices are placed at the interior of the protein shell whereas A and C are turned towards the external surface of the tetracosameric molecule [7]. Following the Drysdale model, the two types of subunits can form 25 different heteropolymers [10]. Each tissue contains molecules with compositions in H:L ratios which are sensitive to tissue iron loading, this usually causes a relative increase in L subunits; whereas, heart, brain and also red cell ferritin are generally H-chain rich [11]. In contrast most bacterial ferritins are generally composed of a single subunit type.

H-chains catalyse the oxidation of Fe(II) to Fe(III) (ferroxidase activity) and the functional importance of L-chains is thought to reside primarily in their ability to promote ferrihydrite nucleation; it has been demonstrated that these two chains have co-operative roles in iron mobilisation [12–15].

We report here a mass spectrometry study of the demetallation of haemin by recombinant horse L-chain apoferritin and its mutant (E 53,56,57,60 Q) in acidic and basic conditions after different times of incubation with haemin at stable temperature.

2. Materials and methods

2.1. Materials

The material use to prepare the recombinant L-chain apoferritin and its mutant (E 53,56,57,60 Q) before incorporation of haemin are: Haemin Chloride Bovine (Sigma–Aldrich, St. Louis, USA), NaN_3 (Merck, Darmstadt, Germany), Casein hydrolysate (Merck, Darmstadt, Germany), Yeast extract (Merck, Darmstadt, Germany), NaCl (Fisher Scientific, London, UK), Ammonium sulphate (Acros Organics, New Jersey, USA), TRIS ultrapure (Applichem Biochemica, Darmstadt, Germany), Sephacryl S-300 (Pharmacia, Uppsala, Sweden), Thioglycolic acid (Acros Organics, New Jersey, USA), NaOH (Merck, Darmstadt, Germany), NH_4HCO_3 (Merck, Darmstadt, Germany) Pyridine (Fluka Chemika), Acetic acid glacial (Fisher Scientific, London, UK), Bradford (Biorad, München, Germany), Dialysis membrane (Medicell International, Ltd, London, UK) and microconcentrators (Vivapore 5 ml, Vivascience, Sartorius group).

2.2. Methods

1. *Expression of recombinant horse L-chain ferritin and its mutant (E 53,56,57,60 Q)*:

The E. coli strain BMH-71-18 was transformed with the plasmid pMK2100 which is the fusion of the cDNA coding for horse L-chain ferritin and a vector pTZ18U. This expression vector controls the expression of the ferritin gene with a promoter tac which is present together with the Amp^r gene in the same plasmid [17].

A bacterial colony containing the appropriate plasmid or 30 μl of the bacterial solution conserved in glycerol at −80 °C was incubated in 70 ml of 2× TY-Amp medium and left shaking at 37 °C overnight. Afterwards, 50 ml of the resulting culture was incubated in 250 ml of

fresh medium at 37°C,with shaking for 24 h. At the end of the growth phase, the culture was kept in an ice bath for 10 min, centrifuged at 10000 rpm for 25 min, resuspended in a solution of NaN_3 0.02% (w/v) and stored at −20 °C.

2. *Purification of recombinant horse L-chain ferritin and its mutant (E 53,56,57,60 Q)*:

The bacterial cells were lysed by sonication on ice. The supernatant, after centrifugation at 10000 rpm for 30 min, was subjected to a thermal denaturation step at 65°C for 10 min in a shaking water bath. After centrifugation at 15000 rpm for 20 min, the supernatant underwent ammonium sulphate precipitation (56% w/v) at 4 °C, and the pellet, after centrifugation at 15000 rpm for 15 min was resuspended in a minimal volume of 25 mM Tris–HCl buffer, pH 7.5, and dialysed against the same buffer during 24 h. Thereafter, the final step in the purification involved chromatography on Sephacryl S-300.

3. *Apoferritin preparation*:

Apoferritin was prepared from recombinant L-chain ferritin and its mutant by dialysing against 0.5% (v/v) thioglycolic acid, pH adusted to 5.5 with NaOH 6 M, for 24 h at room temperature [18]. The excess of thioglycolic acid was removed by dialysing against ammonium bicarbonate 0.5% (w/v) for 24–48 h. After this time, the apoferritin was dialysed against the appropriate buffer before use. In our case, the two buffers used were: $C_7H_9NO_2$ 0.1 M, pH 5, and NH_4HCO_3 0.1 M, pH 8.

4. *Concentration of recombinant horse L-chain apoferritin and its mutant (E 53,56,57,60 Q) and incubation with haemin*:

After determination of the protein concentration by the Bradford reagent [19] the protein was concentrated from 0.5 to 10–20 mg/ml with the Vivapore method 5 ml concentrator (Vivascience, Sartorius Group). After the concentration, the protein was incubated with 12 mol of haemin per molecule of apoferritin (haemin was dissolved in NH_4HCO_3 0.1 M buffer to have a stock concentration of 8 mM).

5. *Mass spectrometry*:

Electrospray ionization (ESI) mass spectra in positive mode were acquired on a time of flight mass spectrometer (microTof, Bruker Daltonics, Bremen, Germany). The instrument was calibrated using a solution of horse heart myoglobin 2 μmol/l in water/acetonitrile 50/50 (v/v), 1% HCOOH. The ESI-source was heated to 200 °C.

Sample solutions (concentration 2 μM) were introduced into the mass spectrometer source with a syringe pump (Cole Parmer, Vernon Hills, Illinois, USA) with a flow rate of 5 μl/min. Scanning was performed on a *m/z* range from 50 to 3000, data were averaged for 1 min and then smoothed using the Gaussian algorithm.

ESI-MS spectrum of native ferritin in non-denaturating conditions was acquired on an Q-TOF II instrument (Waters, Massachusetts, USA). Prior to mass spectrometry experiments, non-volatile salts were removed by exchanging the purification buffer of samples against ammonium acetate (50 mM, pH 7) using Vivaspin microconcentrators (Vivasciences). Ammonium acetate buffer is compatible with both the complex stability and ESI-MS analysis. Samples were continuously infused into the ESI source at a flow rate 5 μl/min, and data averaged for 10 min. The atmospheric pressure/vacuum interface parameters were chosen to ensure that the non-covalent interaction can survive the ionization/desorption process. In particular, high pressure in the interface (7 mbar) for good transmission of large *m/z* ions and high cone voltage (200 V) for good desolvation were required.

Calibration was performed using the ions from multiply charged clusters of CsI on a *m/z* range from 2000 to 16000.

3. Results

3.1. Control experiments

Several control experiments were conducted in order to be sure that the protein has some influence on haemin; with these control experiments, we have demonstrated that the signal of Protoporphyrin IX modified (579.80 Da) appears only when the ferritin is incubated with haemin and not when the ferritin, the haemin and the protoporphyrin IX are alone.

We analysed commercial haemin in a solution of CH_3CN/H_2O (50/50 v/v)/1% HCOOH at several concentrations, the detection limit was determined to be lower than 10^{-7} mol/l (Fig. 2a). The observed charge state is 1^+, and is due to Fe(III) and two negative charges on the porphyrin cycle; the monoisotopic mass is 616.2 Da. Fig. 2b shows the Electrospray mass spectrum (ESI-MS) of haemin at 10^{-6} mol/l, this is in perfect agreement with a theoretical spectrum established by isotopic simulation ($C_{34}H_{32}N_4O_4Fe$) in Fig. 2a.

To be sure that it is not modified by itself, we stocked a solution of haemin for 4 months at room temperature at a dark place and analyzed it again (Fig. 2c). The spectrum is identical to the one of the fresh solution in Fig. 2b. This means that haemin in CH_3CN/H_2O (50/50 v/v)/1% HCOOH solution is stable for several months and that haemin without ferritin does not generate the signal at 579.80 Da.

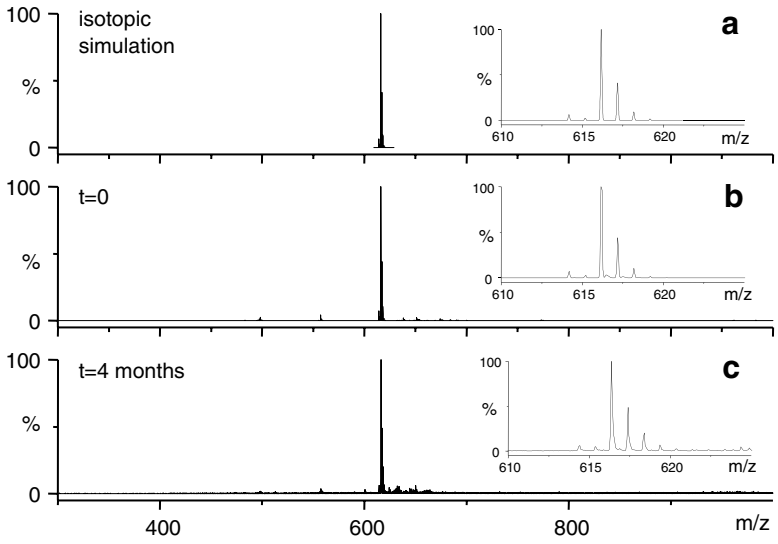

Fig. 2. Control experiment with haemin in CH_3CN/H_20 (50/50 v/v) + 1%HCOOH: (a) isotopic simulation of hemine ($C_{34}H_{32}N_4O_4Fe$) = theoretical spectrum; (b) fresh solution; (c) solution stocked at room temperature for 4 months. The insets are zooms in the *m/z* region of haemin and show the isotopic profiles.

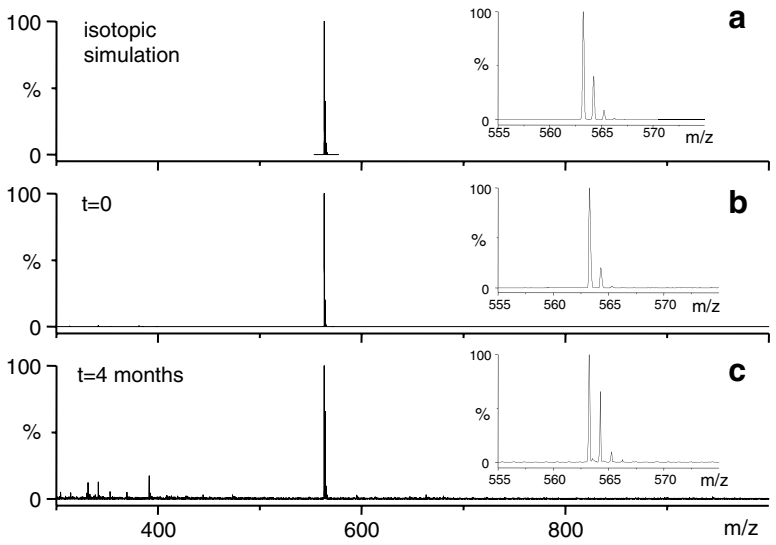

Fig. 3. Control experiment with PPIX in CH_3CN/H_20 (50/50 v/v) + 1%HCOOH: (a) isotopic simulation of protonated PPIX ($C_{34}H_{34}N_4O_4 + H^+$) = theoretical spectrum; (b) fresh solution; (c) solution stocked at room temperature for 4 months. The insets are zooms in the *m/z* region of PPIX and show the isotopic profiles.

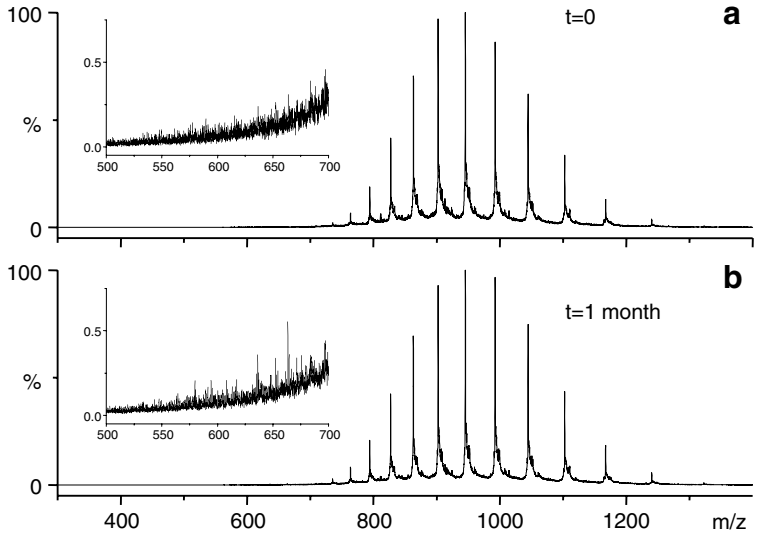

Fig. 4. Control experiment with recombinant ferritin in CH_3CN/H_20 (50/50 v/v) + 1%HCOOH: (a) fresh solution; (b) solution stocked at room temperature for 1 month. The insets are zooms in the *m/z* region of PPIX and haemin.

The same experiment was conducted with commercial Protoporphyrin IX (PPIX) under the same conditions as haemin. PPIX is stable for at least 4 months and both spectra are in excellent agreement with isotopical simulation ($C_{34}H_{34}N_4O_4$+H^+), Fig. 3. PPIX is present in solution in its protonated form, the monoisotopic mass is 563.3 Da. This result means that PPIX does not produce the signal at 579.80 Da.

Finally, another experiment with recombinant ferritin without any addition of haemin or PPIX provided evidence that the protein itself does not generate fragments at low *m/z* values during incubation, Fig. 4. A fresh solution of 10^{-6} mol/l was analyzed and then kept for 1 month at room temperature in a dark place and analyzed again. After this experiment, we can demonstrate that ferritin does not produce the signal at 579.80 Da and that ferritin is a very stable protein (no degradation after a long period of time).

3.2. Ferritin incubated with haemin/PPIX

Ferritin was incubated with haemin and with PPIX in order to get some information if the protein modifies these molecules over a period of 15 days to 12 months.

It was verified by ESI-MS that haemin/PPIX are effectively inserted into the protein: incubated ferritin was dialysed, all the small molecules pass the membrane and only intact protein is retained. After denaturation, haemin/PPIX was detected in the protein solution, whereas in the dialysate no haemin/PPIX could be detected.

That the ferritin molecule was in its intact tetracosahedric form was demonstrated with several techniques: X-ray diffraction, by size exclusion chromatography and by mass spectrometry in non-denaturating conditions.

Size exclusion chromatography was carried out on a Superose 6PC, 30 cm × 3,2 mm column, in 20 mM NH_4HCO_3,

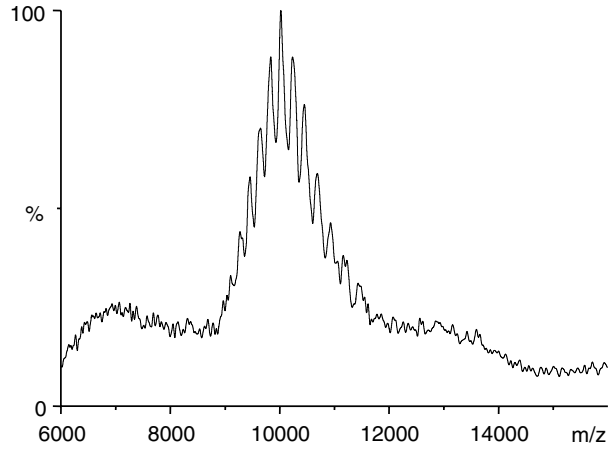

Fig. 5. ESI-MS spectrum of native ferritin in non-denaturating conditions. The signals observed in the region of 10 000 *m/z* correspond to the complex with 24 subunits. Ferritin solution was prepared at 40 μmol/l, in 100 mmol/l pyridinium acetate. The measured mass is 491.469 ± 244 Da.

50μl/min. After calibration of the column with commercial proteins, the molecular weight of the native and the mutant ferritin could be estimated to be about 480 kDa, corresponding to the complex with 24 subunits.

ESI-MS in non-denaturating conditions after desalting of the protein indicated the molecular weight to be 491.469 ± 244 Da, Fig. 5. This high error is due to the low signal obtained in the spectra, and this might be due to a statistical variation in the number of haemin/PPIX molecules inserted into the ferritin. This large error does not allow us to conclude on the presence or not of PPIX/haemin in the protein, but there is no ambiguity that the measured mass corresponds to the ferritin complex with 24 subunits.

High performance liquid chromatography coupled to ESI-MS (HPLC-MS) allowed to confirm that the mutant form of ferritin is correctly mutated: Reversed phase C8 column was used, with a gradient water/acetonitrile, 1 nmol was injected. Four Glu were muted to 4 Gln, and this corresponds to a mass difference of 4 Da for 1 subunit. This mass difference was precisely observed, and allows to conclude that the mutant form is modified in the supposed way. Some other forms were also observed, with a mass shift of 16 Da, and this corresponds to the mass difference expected for oxidation.

Different solutions of apoferritin were incubated with PPIX and haemin, different pH conditions were used: pH 8 (NH_4HCO_3 0.1 M) and pH 5 (pyridinium acetate 0.1 M). Volatile buffers were used because metal salt containing buffers are not compatible with ESI-MS.

At Fig. 6 we present the results obtained with wild type ferritin incubated with haemin in 100 mM NH_4HCO_3 buffer for 15 days and for 12 months (we obtained the same results with the wild type ferritin at pH 5 and with the mutant ferritin (E53,56,57,60Q) at pH 8 and 5). After incubation, the protein was diluted in CH_3CN/H_2O (50/50 v/v) 1% HCOOH to release the incorporated haemin and then analyzed by ESI-MS. The spectrum taken after 15 days shows a strong signal of haemin, whereas in the spectrum after 12 months, this signal has almost disappeared. A doubly charged signal could be detected at 579 *m/z*, which might correspond to protonated, doubly hydroxylated protoporphyrin IX. The isotopical simulation is in perfect agreement with this hypothesis.

4. Discussions

The recombinant wild type horse L-chain apoferritin and its mutant (E 53,56,57,60 Q) have the ability to incorporate and to demetallate haemin to give the hydroxyethyl protoporphyrin IX derivative in a dimeric form (Fig. 7) in acidic and basic conditions and after different times of incubation with haemin (Fig. 8).

The demetallation is faster at acid pH than basic pH. This would have important biological consequences, since we know that ferritin is progressively transformed to haemosiderin within the acidic lysosomal compartment.

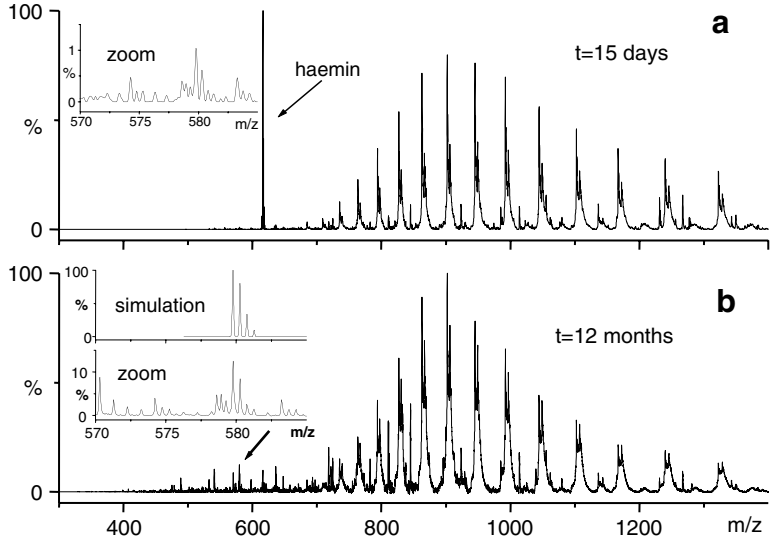

Fig. 6. Ferritin incubated with haemin in CH_3CN/H_20 (50/50 v/v) + 1%HCOOH: (a) solution stocked for 15 days, 12 molecules of haemin/apoferritin; (b) solution stocked for 12 months, 8 molecules of haemin/apoferritin. All solutions were stocked at room temperature. The insets show zooms and in (b) additionally a simulation of the hydroxylated dimer $(C_{34}H_{34}N_4O_4OH)_2 + H^+$.

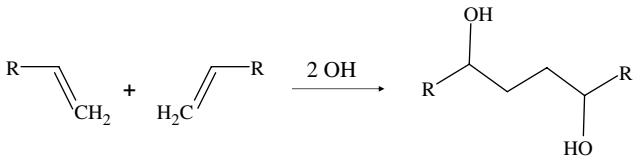

Fig. 7. Formation of hydroxyethyl protoporphyrin IX derivative dimere form.

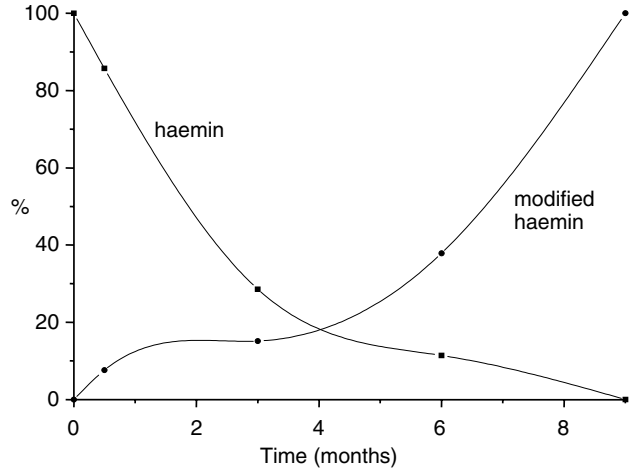

Fig. 8. Intensity of haemin and modified haemin after different times of incubation with recombinant Horse L-chain apoferritin.

The first hypothesis to explain the demetallation implicated the four glutamates situated at the entrance of the hydrophobic pocket. From the present study, we conclude that, while these four glutamates are important – demetallation is faster in the wild type than the mutant – nonetheless their mutation does not stop the incorporation and demetallation of haemin. This suggests that another component of the protein, perhaps arginine 59, which is also located in the hydrophobic pocket, may also be involved.

We are currently constructing two new mutants: R59M and E53,56,57,60Q/R59M to verify the importance of arginine 59 in this process.

Acknowledgments: We thank Professor Louis Hue, Dr. Luc Bertrand and Dr. Noëlle Carette for the construction of protein mutant (E 53,56,57,60Q) and the Université catholique de Louvain for support.

References

[1] Crichton, R.R. (2001) Inorganic biochemistry of iron metabolism. From Molecular Mechanisms to Clinical Consequences, Wiley, Chichester, UK, pp. 326.
[2] Weaver, J. and Pollack, S. (1989) Low Mr iron isolated from guinea pig reticulocytes as AMP-Fe and ATP-Fe complexes. Biochem. J. 261, 787–792.
[3] Fenton, H.J.H. (1894) J. Chem. Soc. Trans. 65, 899–910.
[4] Haber, F. and Weiss, J. (1934) Proc. Roy. Soc. Ser. A 147, 332–351.
[5] Halliwell, B. and Guttheridge, J.M.C. (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem. J. 219, 1–14.
[6] Grossman, M.J., Hinton, S.M., Minok-Bernero, V., Slaughter, C. and Stiefel, E.I. (1992) Unification of the ferritin family of proteins. Proc. Natl. Acad. Sci. USA 89, 2419–2423.
[7] Ford, G.C., Harrison, P.M., Rice, D.W., Smith, J.M.A., Treffry, A., White, J.L. and Yariv, J. (1984) Ferritin: design and formation of an iron-storage molecule. Philos. Trans. R. Soc. Lond. B 304, 551–565.
[8] Chasteen, N.D. and Harrison, P.M. (1999) Mineralization in ferritin: an efficient means of iron storage. J. Struct. Biol. 126 (3), 182–184.
[9] Andrews, S.C., Arosio, P., Bottke, W., Briat, J.F., von Darl, M., Harrison, P.M., Laulhère, J.P., Levi, S., Lobreaux, S. and Yendall, S.J. (1992) Structure, function and evolution of ferritins. J. Inorg. Biochem. 47, 161–174.
[10] Drysdale, J.W. (1977) Ferritin phenotypes: structure and metabolism. Ciba Fdn. Symp. 51, 41–57.
[11] Arosio, P., Adelman, T.G. and Drysdale, J.W. (1978) On ferritin heterogeneity: further evidence for heteropolymers. J. Biol. Chem. 253, 4451–4458.
[12] Levi, S., Yendall, S.J., Harrison, P.M., Santambrogio, P., Cozzi, A., Rovida, E., Albertini, A. and Arosio, P. (1992) Evidence of H- and L-chains have co-operative roles in the iron uptake mechanism of human ferritin. Biochem. J. 288, 591–596.
[13] Santambrogio, P., Levi, S., Cozzi, A., Corsi, B. and Arosio, P. (1996) Evidence that the specificity of iron incorporation into homopolymers of human ferritin L- and H-chains is conferred by the nucleation and ferroxidase centers. Biochem. J. 314, 139–144.
[14] Bauminger, E.R., Harrison, P.M., Hechel, D., Nowik, I. and Treffry, A. (1991) Mossbauer spectroscopy investigation of structure-function relations in ferritins. Biochim. Biophys. Acta 1118, 48–58.
[15] Bauminger, E.R., Treffry, A., Hudson, J.A., Hechel, D., Hodson, N.W., Andrews, S.C., Levi, S., Nowik, I., Arosio, P., Guest, J.R. and Harrison, P.M. (1994) Iron incorporation into ferritins: evidence for the transfer of monomeric Fe (III) between ferritin molecules and for the formation of an unusual mineral in the ferritin of *Escherichia coli*. Biochem. J. 302, 813–820.
[16] Hempstead, P.D., Yewdall, S.J., Fernie, A.R., Lawson, D.M., Artymiuk, P.J., Rice, D.W., Ford, G.C. and Harrison, P.M. (1997) Comparison of the three-dimensional structures of recombinant human H and horse L ferritins at high resolution. J. Mol. Biol. 268, 424–448.
[17] Takeda, S., Otha, M., Ebina, S. and Nagayama, K. (1993) Cloning, expression and characterization of horse L-ferritin in *Escherichia coli*. Biochim. Biophys. Acta 1174, 218–220.
[18] Bryce, C.F.A. and Crichton, R.R. (1973) The catalytic activity of horse spleen apoferritin. Preliminary kinetic studies and the effect of chemical modification. Biochem. J. 133, 301–309.
[19] Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.